GENETIC CHARACTERIZATION OF SINGLE-STRANDED RNA PHAGE

LYSIS GENES

An Undergraduate Research Scholars Thesis

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ABSTRACT

Genetic Characterization of Single-Stranded RNA Phage Lysis Genes

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With an ever-increasing incidence of antibiotic resistance in clinical settings, bacterial viruses, or "phages", are being considered as alternatives to chemical antibiotics. One type of phage in particular, single-stranded RNA (ssRNA) phages, are of interest because they have single gene lysis systems, meaning they employ one gene that, when expressed, causes the host cell to lyse. There are four paradigm single gene lysis systems: *L* from ssRNA phage MS2, A_2 from ssRNA phage Qβ, *E* from single-stranded DNA phage ϕX174, and *Lys* from ssRNA phage M. While the mechanism of L is still unknown, lysis proteins A2, E, and Lys have been shown to inhibit steps in peptidoglycan biosynthesis. It is highly likely that lysis proteins from other ssRNA phages also target this pathway. Previously, however, only fourteen single-stranded RNA phages were known. Two separate papers published within the past year have identified over 150 novel ssRNA phage genomes by mining transcriptome and microbiome data (2, 3). The goal of this project is to identify and test potential lysis genes from these genomes and determine the targets of their lysis proteins. Understanding the mechanisms of these novel lysis proteins would potentially allow us to find new antibiotic targets or even develop new antibiotic strategies.

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ACKNOWLEDGMENTS

NOMENCLATURE

dsDNA   Double-stranded DNA

LB   Lysogeny broth

LPS   Lipopolysaccharide

OD   Optical density

PG   Peptidoglycan

ssDNA   Single-stranded DNA

ssRNA   Single-stranded RNA

CHAPTER I

INTRODUCTION

Overview of bacteriophages

Bacteriophages, literally meaning "bacteria eater" in Greek, are the viruses which infect bacteria. Also called phages, these viruses are ubiquitous in the environment, with the number of phage particles estimated to be 10^{31} in the biosphere and outnumbering bacteria nearly tenfold (4).

Phages were first observed over a century ago by Frederick Twort as round clearings in bacterial growth. Two years later, in 1917, Felix d'Herelle independently observed similar clearings on *Shigella dysenteriae*, the bacteria which causes dysentery, and coined the term bacteriophage (5). At the time antibiotics had not yet been discovered, and d'Herelle was the first to use phage as a therapeutic to treat bacterial disease (6). In fact, despite controversy about d'Herelle's findings in the Western scientific community, phage therapy was used commercially and advertised in medicine up until the advent of penicillin, when it was abandoned nearly everywhere except the Soviet Union (where they used phage as a treatment through World War II) (7). However, phages still had an essential role in basic science, leading to monumental discoveries in molecular biology and genetics.

Max Delbrück, a physicist at Caltech studying the physical nature of the gene, was introduced to bacteriophages as a more convenient model organism than *Drosophila*, and began to work with phage replication, collaborating with fellow researchers Salvador Luria and Alfred Hershey. The three started what came to be known as the Phage Group, offering courses at the

Cold Spring Harbor Laboratory in New York. The research that emerged from this group led to a greater understanding of mutation, the identification of DNA as the hereditary material, the use of phage for genetic recombination, and more (8). More recently, phage display has been instrumental in creating screening libraries in immunology, and CRISPR/Cas systems – a bacterial version of adaptive immunity to protect against repeat phage attacks – have wide future implications in genetic editing.

Phages are entering back into medicine in recent years though, due to the worrying rise in antibiotic-resistant microbes, and researchers have recently found bacterial strains resistant to colistin, a drug of last resort (9). While the need for novel antibiotic classes increases, the development and release of these drugs has slowed considerably. Bacteriophages may be the key to new therapeutic strategies and finding novel antibiotic targets.

Phage morphology

Typically, bacteriophages are characterized into three loose groups: tailed, double-stranded DNA (dsDNA) phages of the order *Caudovirales*; double-stranded, lipid-containing phages; and single-stranded phages with small genomes, a rather diverse category (10). Of the bacteriophages isolated thus far, 96% of them are tailed, dsDNA phages while the remaining 4% is split between the other types of phages (11).

Phages in this group typically have icosahedral capsids or heads encapsulating the DNA, a tail with proteins to facilitate attachment to the host and genome entry, and tail fibers, which also aid in adsorption to the host. This group has been further organized by tail morphology, into three classes. Myophages such as phage T4 have long tails, which contract upon injection of DNA, and siphophages such as phage λ are have long, often flexible tails that are noncontractile.

The third class consists of podophages like P22, which have short tails (12). The genomes of these phages are diverse and range from ~14 kb to ~400 kb, with the exception of Mycoplasma virus P1, a podophage with a genome size of under 12 kb (10).

Lipid-containing bacteriophages consists of four families of phages with diverse morphologies: *Corticoviridae* (icosahedral), *Tectiviridae* (icosahedral), *Cystoviridae* (spherical), and *Plasmaviridae* (pleomorphic). With genomes around 10 to 16 kb in length, these phages all contain lipids, derived from host cytoplasmic membranes and either enclosed within the capsid or form the outer layer of the virion (13).

The single-stranded phages with short genomes has two very distinct phage morphologies. Both ssRNA phages and ssDNA phages in the family *Microviridae* are small, tailless, and have icosahedral heads. Single-stranded RNA phages are the smallest viruses known and have short ssRNA genomes of approximately 4 kb. Similarly, phages in *Microviridae* have small ssDNA genomes of 4 to 6 kb, but encode for spike proteins on the capsid which ssRNA phages do not have. In stark contrast, filamentous phages do not have a capsid, instead consisting of long, thin filaments encasing the circular ssDNA genomes. These phages do not kill their host upon release of virions like the other phages, instead secreting from the membrane.

Bacteriophage life cycle

The bacteriophage life cycle starts with infection, recognizing receptors on the host cell, adsorbing, and then injecting its genome. In stark contrast to a broad-spectrum antibiotic approach, phages interact with specific receptors on bacterial hosts, and slight differences in those receptor proteins – even from one strain to the next – can affect phages' ability to infect

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(14). Because of the difference in chemical composition and structure of the cell wall between Gram-positive and Gram-negative bacteria, phages also adsorb in different manners.

The Gram-negative bacteria cell wall consists of an inner membrane and an outer membrane containing lipopolysaccharides (LPS), with a layer of peptidoglycan in the periplasmic space between the two (15). Peptidoglycan (PG), also known as murein, is a polymer made of glycan strands connected by short peptides and gives the cell shape and mechanical integrity (16). In these Gram-negative bacteria, proteins in the outer membrane, such as transmembrane proteins, as well as components of the LPS can act as receptors for bacteriophages (14).

Gram-positive bacteria, on the other hand, lack an outer membrane and have a much thicker cell wall, composed of peptidoglycan and teichoic acid (15). Phage adsorption on these phages typically rely on components of the teichoic acid formula and acetyl groups in muramic acid, although proteins, likely for transport, in the cell wall of some Gram-positive bacteria can also be phage receptors (14, 17).

Some phages also adsorb to slime or capsular polysaccharides, flagella, or pili of bacteria. Phages such as ΦAcM4 and ΦAcS2 in *Asticcacaulis biprosthecum* have sites connecting the head and tail of the virus to host flagella; since the distant end of the tail remains free to adsorb, it is thought that the phage moves along the flagella towards the cell itself (18). Bacteria also may produce protective layers, such as capsules or slimes, and some phages have adapted to be able to adsorb to antigens or enzymes in these protective layers (14). More well-known are the two types of phages which adsorb to retractable sex pili (19). Filamentous phages adsorb to either F pili, the sex pilus in *Escherichia coli*, produced by the conjugative plasmid F in male

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(F+) strains, or the sex factor *I*, produced by *colI* (20). These phages adsorb to the tip of the pili, preventing conjugation, before their DNA enters the host cell (21). It is proposed that as the pili is retracted into the host, the phage genetic material is also drawn in (22). Single-stranded RNA phages also adsorb to the sex pilus via a maturation protein, and hundreds of virions can actually attach to a pilus at the same time (23). The exact mechanism of RNA injection is unknown, but after absorption, the phage RNA is able to enter the host cell along with the maturation protein (24).

Double-stranded DNA phages can be either virulent or temperate, and they follow different replication strategies. Virulent phages like T4 enter immediately to the lytic cycle, in which the genes needed to produce viral progeny are expressed, and those phage particles are eventually released to the environment upon cell lysis. Temperate phages like λ, however, can enter the lysogenic cycle. When a phage undergoes lysogeny, the phage genome integrates into the host chromosome as a prophage, replicating with the host, and excises under stress conditions, at which point it enters the lytic cycle (25). Through mainly transcriptomic regulation, these phages have the ability to control and time gene expression. Often, they use host machinery to express early genes and begin replication before producing polymerases and other enzymes for transcription and translation of phage DNA (26). This allows for proper timing, so that replication will precede production of phage heads, tails, and tail fibers, which must be able to assemble into progeny with phage DNA packaged before cell lysis (27).

Unlike the dsDNA phages, small lytic phages with single-stranded genomes lack this kind of transcriptional regulation; however, at least for ssRNA phages, it is believed that translation of genes is regulated by the RNA secondary structure (28). Bacterial hosts must also still lyse to release ssRNA phage progeny out into the environment.

Not all bacteriophages lyse the host cell; filamentous phages like coliphage f1 are nonlytic, instead continually producing progeny which extrude out from the membrane into the environment by an unknown process, while the host cell remains alive. However, most phages end their life cycle by somehow disrupting the cell membrane of the bacterial host in order to kill the cell and release virions, and this project focuses on this ultimate step.

Double-stranded DNA phage lysis

In Gram-negative bacteria, double-stranded DNA phages follow a three step lysis process (29). First, Phages form holes in the inner membrane first with a holin, which then leads to the degradation of PG by endolysins, but there are two separate ways this can be achieved. In canonical holin-endolysin lysis, studied in λ, holin proteins accumulate in the inner membrane until triggered, when they aggregate into large rafts and form micron-scale holes (30). The endolysin proteins in the cytoplasm can then escape into the periplasm and start degrading PG (29). Alternatively, the pinholin/SAR endolysin pathway occurs when pinholins, which forms much smaller (around 2 nm), more numerous rafts than the canonical holins, are triggered, depolarizing the membrane (31-33). SAR endolysins, unlike the canonical endolysins, are actually secreted by the host *sec* system and remain in an inactive form, tethered to the inner membrane; this depolarization by pinholins cause the SAR endolysins to change conformation, becoming active, released from the inner membrane (34). However, the outer membrane remains intact, preventing the cell from lysing. This is remedied with spanin proteins, which are proposed to fuse the inner and outer membranes together, and are either unimolecular (u-spanins) or two-component (i-spanins and o-spanins) (35). The lysis genes encoding for these proteins are often, but not always, close together on the phage genome, forming a cassette (36).
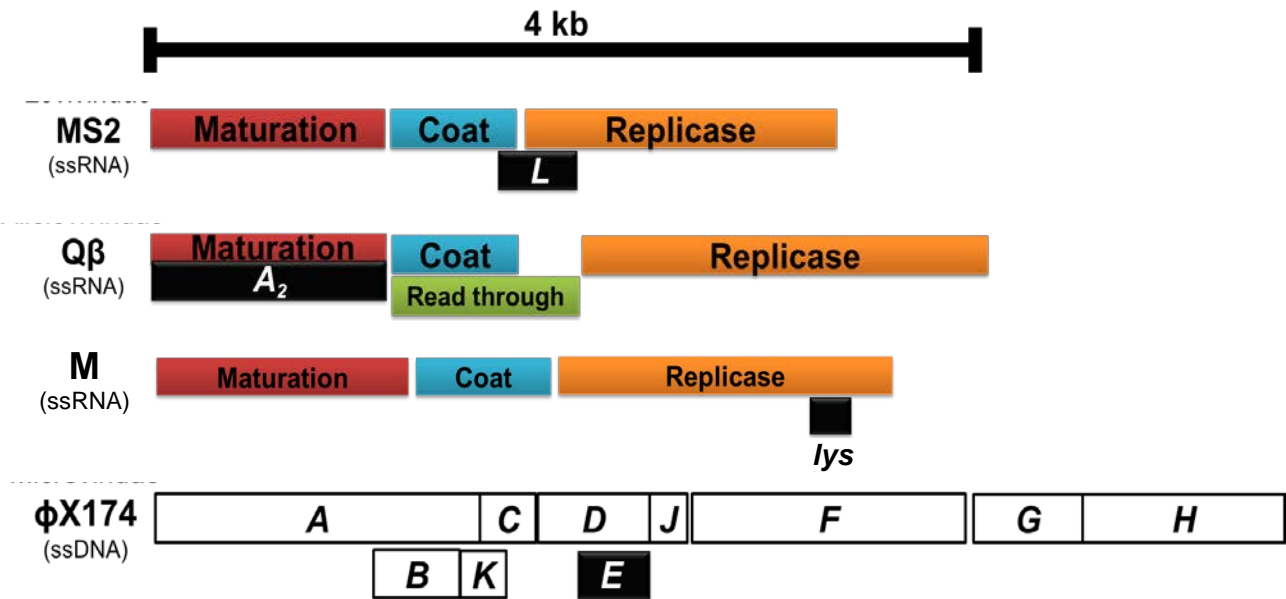
Figure 1 Genome organization of the paradigm phages with single gene lysis systems

Single-stranded RNA phage lysis

Unlike large double-stranded DNA phages which encode three to six lysis proteins, these phages – along with some single-stranded DNA phages – have single gene lysis systems, in which the product of just one gene causes lysis the bacterial cell (37). The interaction between the one phage lysis and a target in the bacterial host will cause cell death and lysis, which could potentially open the door to identifying new targets for antibiotics.

Each ssRNA phage has three core genes, *mat*, *coat*, and *rep*, coding for the maturation protein which recognizes the sex pilus, coat protein which composes the capsid, and an RNA-dependent RNA polymerase, respectively. Although these genes have high sequence homology from one ssRNA phage to the next, the lysis gene varies greatly and has almost no similarity between phages. There are four paradigm single gene lysis systems, for which the lysis mechanism is known for three: A_2 from ssRNA phage Qβ, Lys from ssRNA phage M, and E from ssDNA phage φX174 (Fig 1). The lysis proteins from these three phages inhibit enzymes

involved in the biosynthesis of peptidoglycan (Fig 2). Lysis protein A_2 from Qβ inhibits MurA, which catalyzes the first step in PG biosynthesis pathway, while E from φX174 inhibits MraY, which forms the first lipid-linked intermediate in the pathway (38, 39). Lys, the lysis protein from M, was recently found to block MurJ, an enzyme which flips peptidoglycan from the cytosol into the periplasm (unpublished data). The fourth paradigm single gene lysis system is L, discovered in 1979, from ssRNA phage MS2 (40). Despite the studies performed with MS2 – most recently focusing on its structure, RNA encapsidation, and potential as a scaffold for drug delivery – the lytic function of MS2 has still not been determined (41, 42). Unlike A_2, Lys, and E, which cause septal collapse, L produces random blebbing, a different lysis phenotype. With septal collapse, blebbing occurs at the mid-cell region where the septum forms during cell
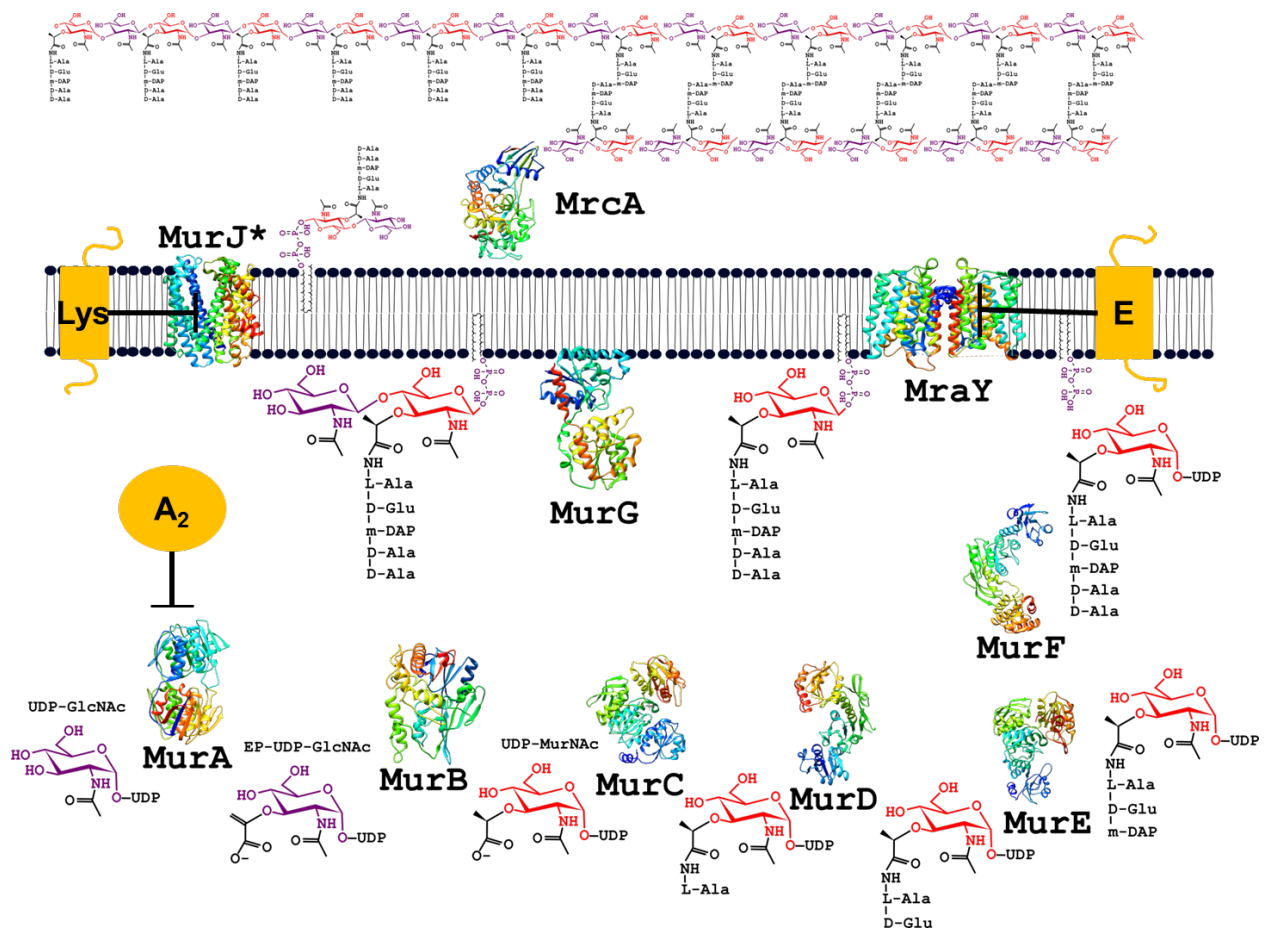

Figure 2 Peptidoglycan biosynthesis pathway, with ssRNA phage lysis proteins known to inhibit enzymes in the pathway shown. Enzymes shown were created with UCSF Chimera (1).

division, while in random blebbing, as the name suggests, blebs are inconsistent, forming in various regions around the cell. This suggests L may target an entirely different pathway, but the lysis mechanism of L has remained unsolved for nearly 40 years.

Novel ssRNA phage genomes

Only fourteen ssRNA phages and 3 ssDNA phages with these single gene lysis systems were previously known, limiting the studies of their diverse lysis mechanisms. However, over 150 novel phage genomes were isolated from transcriptomic and environmental metadata within the past year (2, 3). The phage genomes were identified through sequence homology of the three core genes, but just one of those genomes had an annotated lysis gene, a cistron that was 40% similar to but much longer than MS2 *L* (2). This is in part because of the extremely high mutation frequency; the genetic architecture (i.e., sequence, size and position within the genome) of the lysis gene varies greatly from phage to phage, and homology between lysis genes is uncommon. As a result, a new bioinformatics approach must be taken to identify novel lysis genes. In addition, the lysis genes from these ssRNA phages may provide insight into novel ways to kill bacteria or help find new antibiotic targets.

CHAPTER II

METHODS

Culture growth and bacterial strains

Except when specified, LB and agar were used as the growth medium. Antibiotics used when appropriate were ampicillin (Amp) at 100 ug/mL, kanamycin (Kan) at 40 ug/mL, chloramphenicol (Cam) at 10 ug/mL, and tetracycline (Tet) at 10 µg/mL. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), Isopropyl β-D-thiogalactopyranoside (IPTG), and arabinose were added as needed at a final concentration of 1 mM, 10 µg/ml and 0.4%, respectively. *E. coli* XLI-Blue was the bacterial strain used in these experiments, unless otherwise indicated.

Annotation of lysis gene candidates

Novel ssRNA phage sequences discovered by Krishnamurthy et al. and Shi et al. were manually annotated for potential lysis genes using Artemis, a genome browser and annotation tool from the Sanger Institute. The three core genes, *mat*, *coat*, and *rep*, were previously annotated based on sequence homology (2, 3). Lysis gene candidates were selected based on TMHMM analysis and the Shine-Dalgarno sequence, the ribosomal binding site, of open reading frames greater than 25 nucleotides.

Synthesis of lysis genes and cloning

Lysis gene candidates, flanked by EcoRI and XhoI restrictions sites, with a HindIII site a few nucleotides downstream, were constructed in groups of gBlocks from Integrated DNA

Technologies. GBlocks from Integrated DNA Technologies were also used to synthesize modified lysis genes for added protein tags, linkers, or nucleotide changes in the lysis proteins.

For newly synthesized lysis gene candidates, a *lacZα* gene was inserted using XhoI and HindIII sites. EcoRI and HindIII sites were used to insert genes into an ampicillin-resistant pBad24 vector. Enzymes were purchased from New England Biolabs, and ligation was performed following manufacturer instructions. Plasmids were transformed into bacteria using standard methods.

Testing potential lysis genes

Bacteria with inserted plasmids were streaked simultaneously on LB inducer plates (Amp and arabinose) and non-inducer (Amp) plates. DNA from colonies on the non-inducer plates, which also showed growth defects on inducer plates, was extracted using the Qiagen Miniprep protocol and sequenced through Eton Biosciences. A modified blue-white suppressor screen, in which the plasmid vector carried a *lacZω*, while the insert carried *lacZα,* was used to ensure presence of the inserts through blue colonies.

Overexpression of MurJ and ectopic expression of *Caulobacter crescentus* MraY were achieved using expression from Amp-resistant pCM6 and Cam-resistant pZA22 vectors, respectively. Lysis genes were also expressed in *E. coli* strain TB28 with *mraY* replaced with the homologous gene from *Caulobacter crescentus*.

Growth curves

Selected colonies were grown at 37°C in 5 mL overnight cultures. Then, 25 mL cultures of LB supplemented with 100 ug/mL ampicillin were inoculated with a 1:200 dilution of the

overnight cultures and grown at 37°C. The culture growth and lysis were monitored at an absorbance of 550 nm (A_{550}) as previously described (43). At time 0, when A_{550} ~0.2, the cultures were induced with arabinose. Absorbance measurements were taken at specific time intervals post induction.

CHAPTER III

RESULTS

Genomes annotated for lysis gene candidates

Potential lysis genes were annotated in 90 out of the 188 novel ssRNA phage genomes

previously identified, yielding 126 lysis gene candidates (2, 3). Multiple novel genetic

architectures were found in terms of the location of the lysis gene, e.g. in the +1 reading frame of

coat or partially embedded in *rep* (Fig. 3). These genes were manually annotated based on open

reading frames greater than 25 codons with strong Shine-Dalgarno sequences and

transmembrane domains. While not all ssRNA phage lysis proteins are transmembrane, most of

the lysis genes from previously known ssRNA phages have predicted transmembrane domains,

allowing us to use this parameter to narrow down potential candidates. That said, 5 of the

genomes annotated had no good candidates with a transmembrane domain, and the annotated
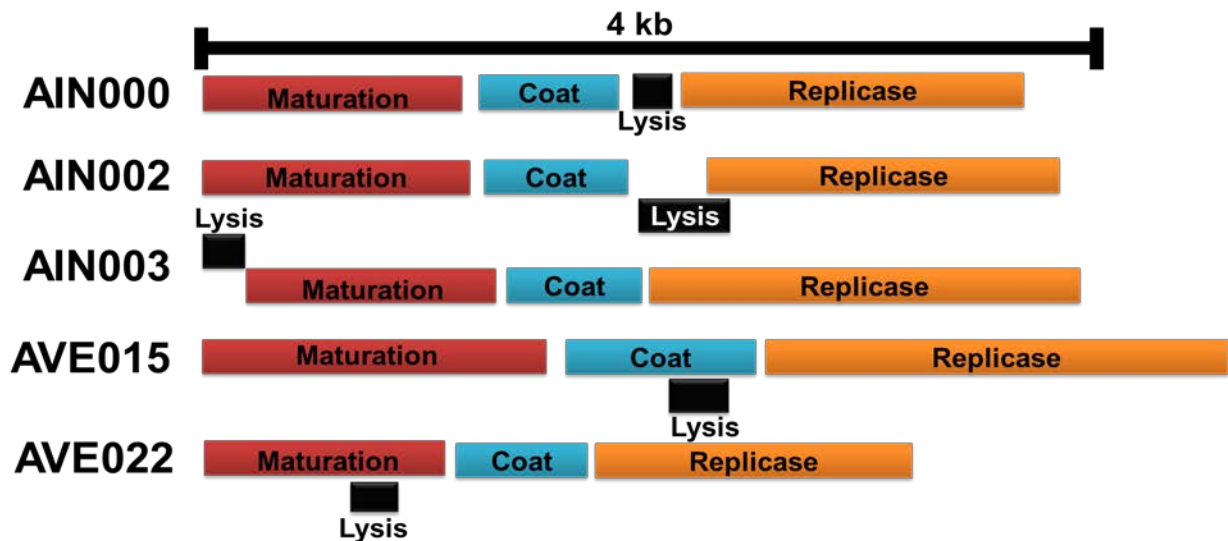

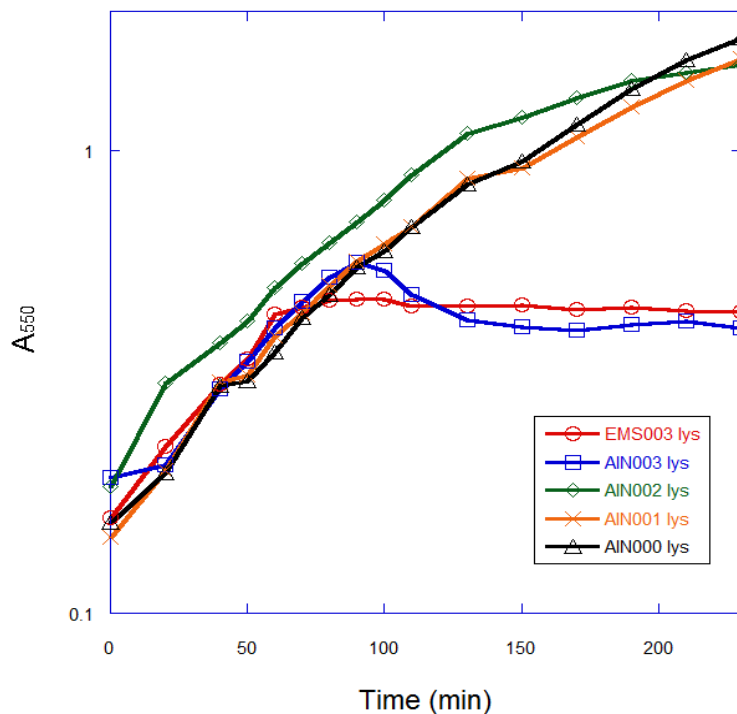**Figure 3** Examples of novel genetic architectures found.

Figure 4 Lysis curves of *E. coli* with novel lysis genes expressed

lysis gene candidates had strong other indicators, like a good start codon and ribosomal binding site.

Novel lysis genes produce growth defects or lysis

Furthermore, 55 of the lysis gene candidates were synthesized and expressed in *E. coli;* five were shown to cause growth defects or lysis on plates. However, when growth curves were done in liquid media, two showed partial lysis (AIN003 *lys* and EMS 003 *lys*) while the remaining three did not show any growth defect (Fig. 4). EMS003 *lys* is embedded in the +1

Figure 5 Amino acid sequence of lysis proteins from EMS003 and AIN003. Predicted transmembrane domains are underlined, and positively and negatively charged amino acids are highlighted in blue and red, respectively.

reading frame of the replicase gene and encodes for a 52-amino acid protein with a predicted transmembrane. AIN003 Lys also has a predicted transmembrane domain, but is only 27 amino acids in length, shorter than any previously known lysis protein; the gene itself is separate, upstream of the maturation protein (Fig. 5).

EMS003 Lys targets *Caulobacter crescentus* MraY

Although EMS003 Lys shows partial lysis in *E. coli*, it is similar in terms of genomic architecture to ΦCb5, a *Caulobacter crescentus* phage; both lysis genes are in an embedded +1 reading frame of the replicase gene (44). In addition, the lysis protein of ΦCb5 is similar to that of E, which targets *E. coli* MraY. Because the original host of the phage EMS003 is unknown, EMS003 was tested in an *E. coli* strain with MraY replaced with the MraY from *C. crescentus*. When EMS003 *lys* was expressed in this strain, there was complete lysis in comparison to the partial lysis originally seen (Fig. 6). This suggests that EMS003 *lys* targets *C. crescentus* MraY, and that *C. crescentus* may be the phage's original host.
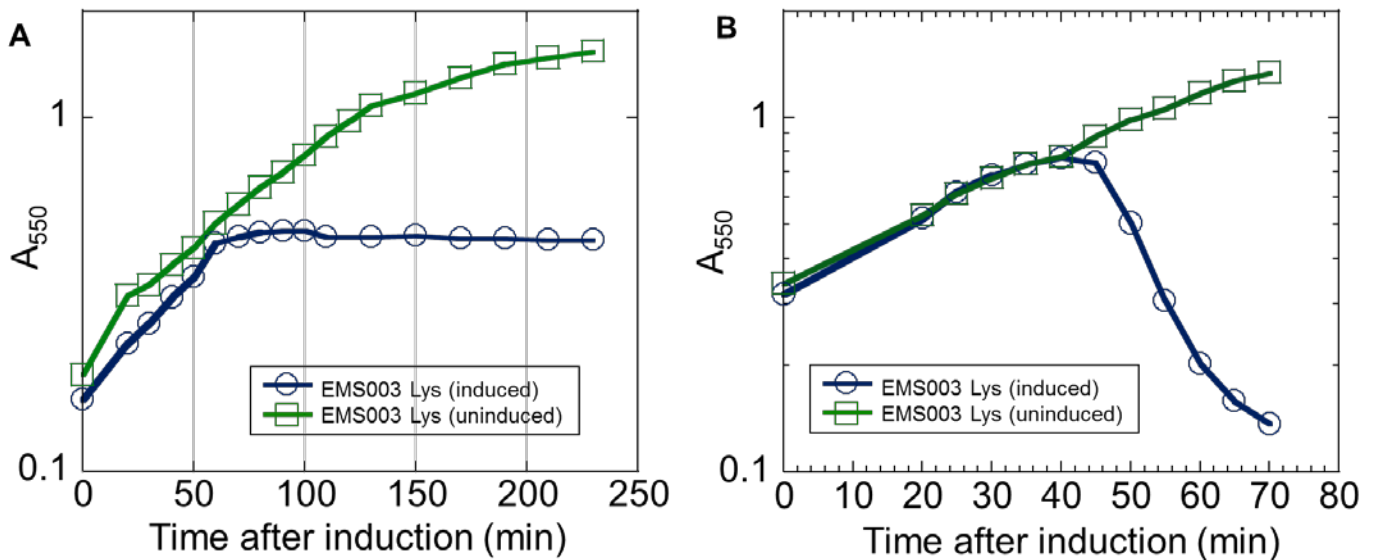

Figure 6. (A) Lysis curve of EMS003 lys expressed in *E. coli* XLIBlue strain, with uninduced culture as negative control. (B) Lysis curve of EMS003 *lys* expressed in *E. coli* strain TB28 with a CcMraY::Kan gene replacement, with an uninduced culture as negative control.
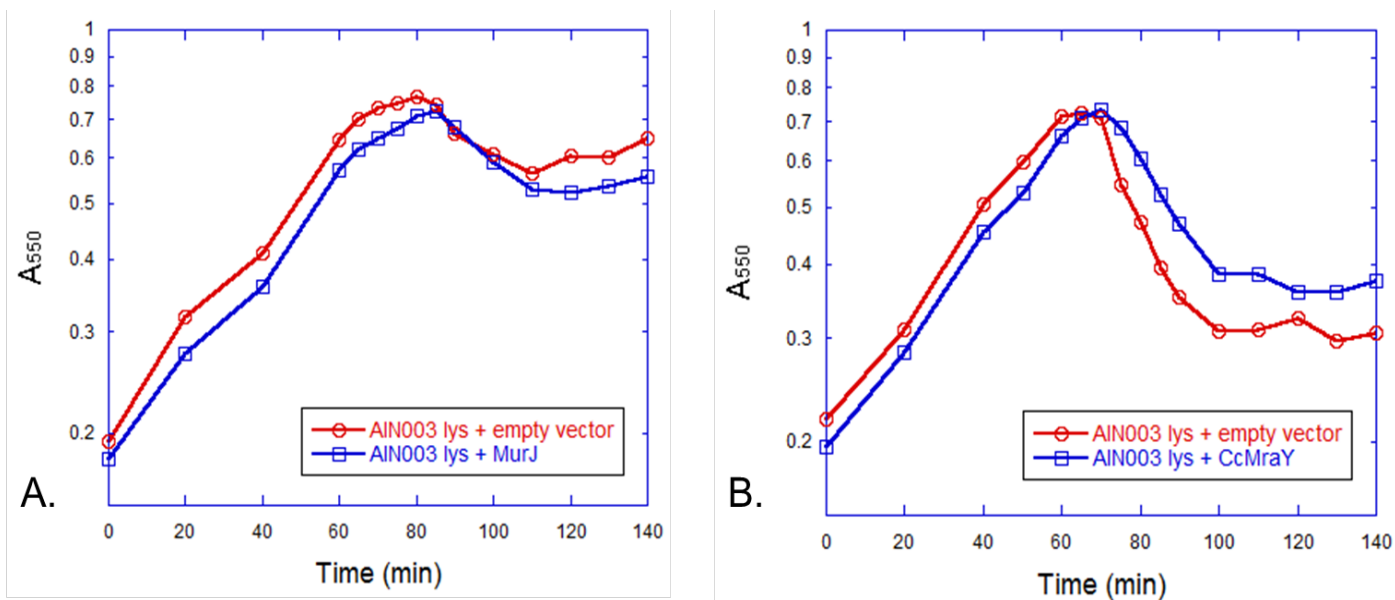
Figure 7. (A) Lysis curve of AIN003 lys with MurJ overexpressed on a plasmid, and negative control. (B) Lysis curve of AIN003 lys with *C. crescentus* MraY expressed on a plasmid, with negative control.

AIN003 Lys does not target MraY or MurJ

In contrast, AIN003 Lys does not target either transmembrane protein known to be part of the PG biosynthesis pathway. Overexpression of MurJ has been shown to rescue bacteria from lysis caused by Lys from phage M (unpublished data). However, when MurJ is overexpressed with AIN003 Lys, lysis still occurs (Fig. 7a). Similarly, the expression of MraY protein from *Caulobacter crescentus* has been shown to rescue lysis from E, from ΦX174 (unpublished data). However, this also did not rescue lysis from AIN003 Lys (Fig. 7b).

CHAPTER IV

CONCLUSION

Because so few ssRNA phages were previously known, the identification of so many novel ssRNA phage genomes in the past year opens the door for studying the diversity of these viruses' lysis genes. The lack of sequence similarity and placement within the genome of these lysis genes hint at a range of lysis mechanisms, which may provide novel antibiotic strategies or locate a new target for antibiotics.

Lysis gene candidates that were annotated were open reading frames greater than 25 codons and had a strong ribosomal binding site and predicted transmembrane domain, like lysis genes already known. However, it is highly likely that there are lysis proteins from these phages that are soluble (like A_2 from Qβ), especially as at least five of the genomes had potential lysis gene candidates without a transmembrane domain, based on near-consensus Shine-Dalgarno sequences and strong probabilities out of all open reading frames in their respective genomes.

Although we only identified two lysis genes that showed a lysis phenotype in liquid culture, it is critical to note that the original hosts of the novel phages remain unknown as they were pulled from environmental metadata. The lysis gene candidates annotated were only expressed in *E. coli*, and those negative for lysis may likely be from a different host.

EMS003 Lys, even though it targets MraY from *C. crescentus*, like ΦX174 E in *E. coli*, the two lysis genes share no significant sequence similarity, and likely interact with the target in differing ways. AIN003 Lys, despite having a predicted transmembrane domain, does not target the two known transmembrane proteins in the PG biosynthesis pathway, suggesting either that

the protein itself is soluble despite the strong prediction or that it targets an entirely different pathway or protein. This is of considerable interest as there is no single lysis protein yet identified that can kill and lyse the bacterial cell through a mechanism outside of PG biosynthesis.

Future goals include annotation of more of the recently identified ssRNA phage genomes, and synthesizing lysis gene candidates from those genomes. Any novel lysis genes found in *E. coli* will be tested in a comparable manner to EMS003 *lys* and AIN003 *lys*. Further investigation into the *C. crescentus* MraY – EMS003 Lys interaction will be done as well. Steps will be taken to achieve better lysis with AIN003 Lys.

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